

Dissecting the expression dynamics of RNA-binding proteins in posttranscriptional regulatory networks

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Edited by Michael Levitt, Stanford University School of Medicine, Stanford, CA, and approved October 5, 2009 (received for review June 22, 2009)

In eukaryotic organisms, gene expression requires an additional level of coordination that links transcriptional and posttranslational processes. Messenger RNAs have traditionally been viewed as passive molecules in the pathway from transcription to translation. However, it is now clear that RNA-binding proteins (RBPs) play an important role in cellular homeostasis by controlling gene expression at the posttranscriptional level. Here, we show that RBPs, as a class of proteins, show distinct gene expression dynamics compared to other protein coding genes in the eukaryote *Saccharomyces cerevisiae*. We find that RBPs generally exhibit high protein stability, translational efficiency, and protein abundance but their encoding transcripts tend to have a low half-life. We show that RBPs are also most often posttranslationally modified, indicating their potential for regulation at the protein level to control diverse cellular processes. Further analysis of the RBP-RNA interaction network showed that the number of distinct targets bound by an RBP (connectivity) is strongly correlated with its protein stability, translational efficiency, and abundance. We also note that RBPs show less noise in their expression in a population of cells, with highly connected RBPs showing significantly lower noise. Our results indicate that highly connected RBPs are likely to be tightly regulated at the protein level as significant changes in their expression may bring about large-scale changes in global expression levels by affecting their targets. These observations might explain the molecular basis behind the cause of a number of disorders associated with misexpression or mutation in RBPs. Future studies uncovering the posttranscriptional networks in higher eukaryotes can help our understanding of the link between different levels of regulation and their role in pathological conditions.

disease | posttranslational modifications | protein noise | regulation | systems biology

Gene expression is a highly regulated process and is controlled at several levels. In eukaryotes, control of gene expression first occurs at the level of transcription, where transcription factors regulate the synthesis of RNA of specific genes in response to different internal and external stimuli. On the other hand, at the protein level, several posttranslational modifications, such as phosphorylation by kinases and ubiquitin ligases, are known to spatially and temporally control the availability of functional protein products within the cell. However, a much less understood level of gene expression regulation, which occurs between these two layers, is due to the posttranscriptional control of RNAs. It is now increasingly known that this level is controlled by numerous factors with major players being the RNA-binding proteins (RBPs) (1–3) (see Fig. 1A). Therefore, intricate coordination of regulation from these three different layers is important for finely controlling the flow of genetic information from genes to proteins in different conditions. Indeed, changes in gene expression due to aberrations at any of these three levels have been shown to be responsible for the cause of a number of disorders (4–8).

Development of DNA microarray technology has made it possible to measure the expression of each annotated gene at the transcript level. Indeed, this technique has been the high-throughput approach of choice to efficiently characterize the transcriptomes of several model organisms. One common assumption

in DNA microarray experiments is that the level of mRNA of a particular gene reflects the amount of protein and there is little regulation at the posttranscriptional level. Recent studies comparing the high-throughput data for mRNA and protein abundances indicate that there is a very weak correlation between the number of transcripts and protein products of a gene, challenging this notion (9, 10). This suggests that the regulation of gene expression at the posttranscriptional level is predominant. For instance, in the eukaryotic pathogen, *Trypanosoma cruzi*, it is well known that gene expression is primarily controlled at the posttranscriptional level through RNA-binding proteins (RBPs) (11). These studies suggest the extensive role of posttranscriptional regulation in controlling gene expression in eukaryotes (12, 13).

In eukaryotes, transcription and translation occur in different compartments. This allows for a plethora of options to control RNA at the posttranscriptional level, including their splicing, polyadenylation, transport, mRNA stability, localization, and translational control (2, 3). Although some early studies revealed the involvement of RBPs in the transport of mRNA from nucleus to the site of their translation, increasing evidence now suggests that RBPs regulate almost all of the posttranscriptional steps shown in Fig. 1A. For example, in humans, Nova protein is associated with splicing (14); PUF family proteins have been shown to play an important role during *Caenorhabditis elegans* oogenesis (15); Tap protein, like its yeast homolog Mex67, was reported as a bona fide mRNA nuclear export factor (16); Puf3p in yeast was shown to be responsible for localization of mitochondrial transcripts (17); and Pab1 was reported to regulate the initiation of translation (18). While the extensive role of RBPs in posttranscriptional control of cellular processes has been reviewed by several groups (1–3, 7), in yeast alone we found that RBPs are involved in multiple cellular processes and components (see *Materials and Methods* and *SI Text S1*). All these aspects highlight the importance of RBPs in regulating gene expression at the posttranscriptional level.

Due to their central role in controlling gene expression at the posttranscriptional level, alteration in expression or mutations in either RBPs or their RNA targets (i.e., the transcripts which physically associate with the RBP) have been reported to be the cause of several human diseases such as muscular atrophies, neurological disorders, and cancer (6, 7, 19, 20). In particular, disorders such as myotonic dystrophy (DM) and oculopharyngeal muscular dystrophy (OPMD) have been attributed to RNA's gain-of-function by CUG repeat expansion in the case of myotonic dystrophy protein kinase (DMPK) (19) and GCG repeat expansion in exon 1 of the RBP, PABPN1 in the case of OPMD (7), respectively. On the other

Author contributions: S.C.J. designed research; N.M. performed research; N.M., N.R., M.M.B., and S.C.J. contributed new reagents/analytic tools; N.M. and S.C.J. analyzed data; and N.M. and S.C.J. wrote the paper.

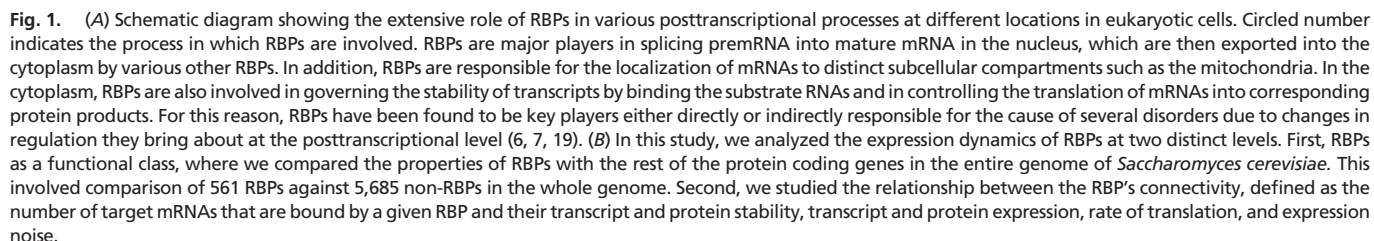
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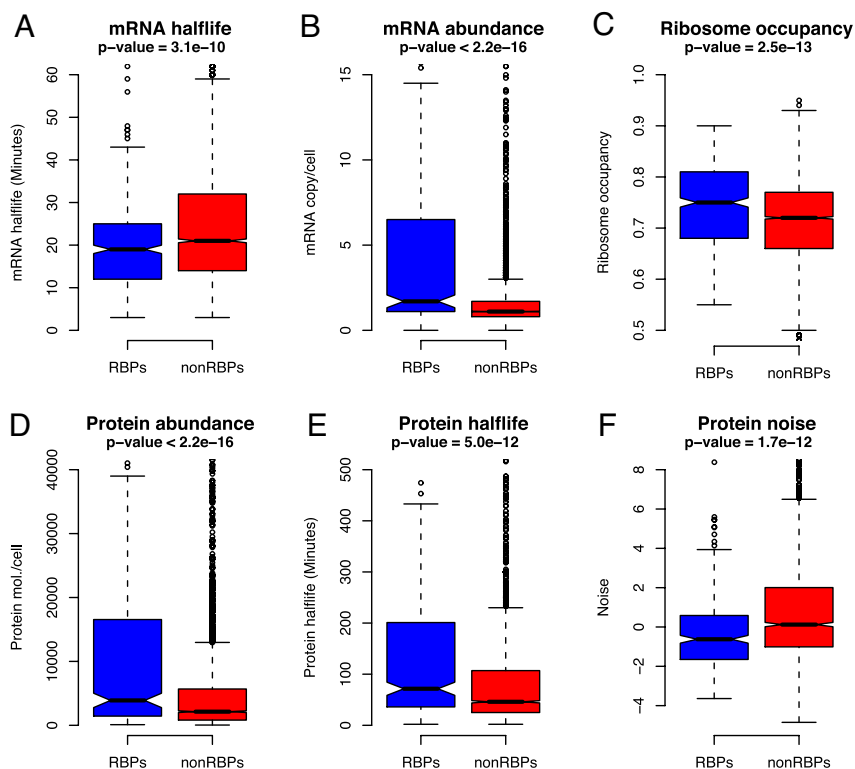
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This article contains supporting information online at www.pnas.org/cgi/content/full/0906940106/DCSupplemental.



Ribosome occupancy has been shown to be a measure of translational efficiency of mRNA. Higher ribosome occupancy relates to higher protein synthesis, and lower ribosome occupancy indicates low translation rate of mRNA. We next asked whether the ribosome occupancy i.e., rate of translation, of RBPs is higher than those for non-RBPs and whether their protein levels are higher



within the cell. This analysis clearly revealed that RBPs have high ribosome occupancy ($P = 2.5 \times 10^{-13}$, Wilcoxon test) (Fig. 2C) and are also present in much higher concentrations ($P < 2.2 \times 10^{-16}$, Wilcoxon test) (Fig. 2D) with median abundances of RBPs being roughly double that observed for non-RBPs (3,895 versus 2,132 protein molecules/cell). These results indicate that RBPs are abundant and are translated rapidly, supporting the versatile nature of their involvement in multiple posttranscriptional control mechanisms at different cellular locations (*SI Text S1*). Exclusion of ribosome and translation-associated factors from RBPs to compare nonribosomal RBPs against non-RBPs indicated that ribosomal RBPs contribute significantly to the observed differences in the rate of translation and protein abundance of RBPs (*SI Text S2*). Comparing the protein concentrations of nonribosomal RBPs with non-RBPs indicated that the former are still significantly more abundant ($P = 2.2 \times 10^{-2}$).

Stability of a protein measured as its half-life can be considered as a proxy for the life time of a protein in a cell. Therefore, to understand the degradation rates of RBPs and to compare them against non-RBPs we analyzed their protein half-lives (see *Materials and Methods*). This analysis revealed that RBPs are significantly more stable than non-RBPs, with RBPs exhibiting a median half-life of 71 min as against non-RBPs with 46 min ($P = 5 \times 10^{-12}$, Wilcoxon test) (Fig. 2E). Repeating the analyses with nonribosomal RBPs showed a consistent trend despite their exclusion ($P = 4.8 \times 10^{-2}$) (SI Text S2). Our observations on the increased protein stability and concentration of the RBPs compared to other proteins in the cell suggests that RBPs, whose main functional role is in the processing and localization of their mRNA targets, might be required at multiple subcellular locations and be used throughout the cell cycle. This may likely warrant their higher abundance and stability at the protein level. It is important to note that although RBPs exhibit high protein stability, they also show low transcript stability, which indicates that most RBPs that are stable at the protein level, might be avoiding cellular crowding of their transcripts by quick turnover at the transcript level. Indeed, it has been

shown in yeast that most RBPs autoregulate their own activity at the transcript level (23).

To understand how these properties vary with different processes in which RBPs are involved, we divided RBPs into four major categories: translation, transport, RNA localization, and processing using GO annotations and compared them with non-RBPs (*SI Text S3*). This analysis revealed that the general trends observed for different categories are similar to those seen for RBPs as a whole, although certain categories comprised relatively few RBPs.

Several RBPs have been shown to be posttranslationally modified, which adds a layer of flexibility to their function. Many of these posttranslational modifications have been shown to modify their RNA-binding properties or their subcellular localization. Indeed, at least four types of posttranslational modifications namely phosphorylation, ubiquitination, methylation, and SUMOylation have been reported for RBPs (2). High stability of RBPs indicates the potential that posttranslational modifications can offer in the diversification of their function. In fact, analysis of the number of kinase substrates in RBP and non-RBP populations using the currently available protein phosphorylation map for yeast (24), suggests that some kinases not only target higher number of RBPs compared to non-RBPs ($P = 2.7 \times 10^{-2}$) but also more kinases are associated with RBPs ($P < 2.2 \times 10^{-16}$) (see *SI Text S4*).

Gene expression is a highly dynamic process and because of its dynamic nature there is a large variation in a protein's abundance among different cells in a population. This variation is termed as biological noise. Genes whose expression varies to a large extent show more noise and these are typically involved in stress response, amino acid biosynthesis, and heat shock. On the other hand, genes that show consistent expression during the cell cycle such as those involved in protein degradation and ribosomal proteins tend to show low noise (25). Here, we have explored this noise data, to address whether RBPs show significant difference from non-RBPs in terms of biological noise. As shown in Fig. 2*F*, RBPs were found to show significantly lower noise levels in comparison to non-RBPs ($P = 1.7 \times 10^{-12}$, Wilcoxon test). Reanalyzing the data by excluding ribosomal proteins still clearly indicated that RBPs exhibit much

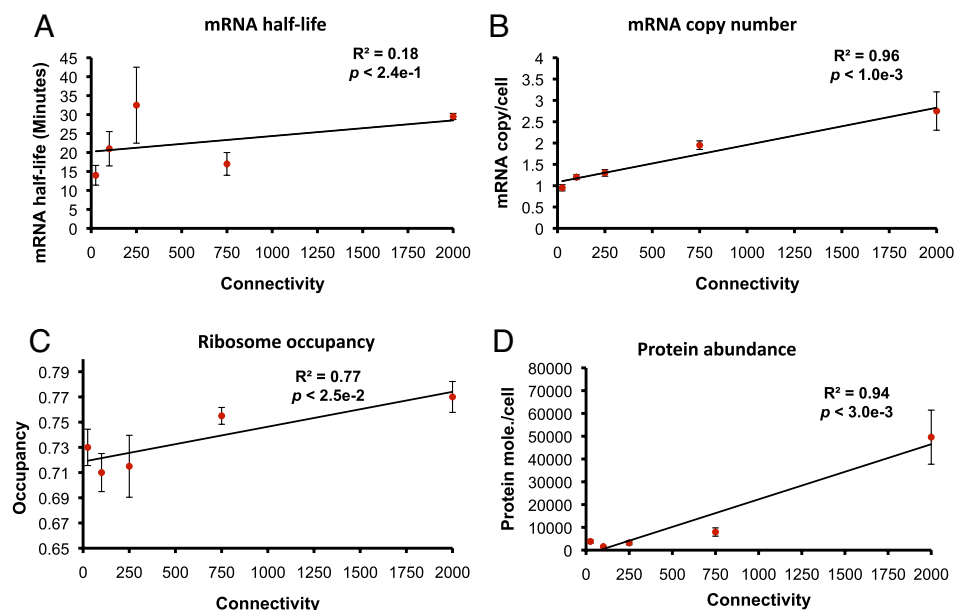


Fig. 3. Relationship between the number of targets of a RBP and its (A) transcript turn over, (B) estimated mRNA copy number per cell, (C) extent of ribosome occupancy, and (D) protein abundance. In each case, except for transcript stability, we found a strong correlation between the connectivity of a RBP and the regulatory property studied, suggesting that RBPs that regulate high numbers of targets are present at higher levels at the protein level. RBPs are divided into five bins, with approximately equal number of RBPs, based on their connectivity. Points correspond to the median values in the respective bins whereas the error bars show the normalized median deviation calculated as the ratio between the median absolute deviation (MAD) and the square-root of the number of values in the bin.

lower noise compared to other protein coding genes ($P = 6.3 \times 10^{-6}$, Wilcoxon test) (*SI Text S2*). This analysis unambiguously reveals that low noise is an inherent property of all RBPs and suggests that RBPs are tightly regulated at the protein level with little variation in their expression from cell to cell. An independent analysis to compare the dynamic properties of RBPs with all of the protein coding genes (including RBPs) and varying the test statistic used to calculate the significance, did not change our results. This suggests that the trends observed are generally robust and are independent of the statistical test used (*SI Text S5*).

The Number of Distinct Targets Bound by a RBP Is Correlated with Its Cellular Abundance. RBPs are the key elements responsible for the posttranscriptional control of gene expression and when combined with their RNA targets, this information can be represented as a RBP-RNA network. Although, on a genomic scale, RBPs are believed to control diverse range of functions with some eukaryotic systems predominantly using posttranscriptional mechanisms for gene expression control (11, 13), large-scale elucidation of posttranscriptional networks is limited to few model organisms for a select set of RBPs. In yeast, few recent genomewide studies identified the targets for several RBPs using RIP-chip technology (23, 26). These studies revealed the important roles played by different families of RBPs and the structure of the posttranscriptional network formed by them. These high-throughput studies showed that the number of targets of a RBP can vary widely, from <10 to more than thousands. In this study we obtained this network, where nodes represent RBPs or their targets and links represent a distinct physical association between the RBP and the target RNA. We then systematically investigated the relationship between different dynamic properties of RBPs and the number of distinct RNA targets they control.

We first asked whether the number of targets of a RBP is correlated with its transcript stability by grouping the RBPs into different connectivity bins i.e., groups of RBPs comprising a number of distinct RNA targets (see *Materials and Methods*). As a result of this analysis, we found that there was a weak but positive correlation between them, suggesting that transcript turnover of RBPs may not be dependent on their number of targets ($R^2 = 0.18$, $P < 0.24$) (Fig. 3A). On the other hand, a comparison of the mRNA copy number of a RBP and its number of targets revealed a strong positive correlation between them, suggesting that RBPs with a high number of targets are likely to be more highly expressed at the

mRNA level ($R^2 = 0.96$, $P < 1 \times 10^{-3}$) (Fig. 3B). For instance, PAB1 is a highly connected essential RBP that can bind to the poly(A) tail of an mRNA to regulate its translational initiation through its binding with eIF4G protein (18, 27). Indeed, it was reported to bind to 1,994 distinct RNA targets and was among the genes with very high mRNA copy number (7.1 mRNA copies/cell). These observations point to a direct link between the number of distinct targets of a RBP and its available number of copies of mRNA in the cell. To test the existence of a correlation between the connectivity and the rate of translation or the absolute protein abundance profile of RBPs, we further explored the relationship between them (Fig. 3C and D). This comparison uncovered a more general link between translational efficiency of a RBP and its degree. For instance, Pub1p is another poly(A) binding protein (28) that binds to diverse sets of transcripts involved in ribosome biogenesis, cellular metabolism, and transport (29). This protein was reported to be localized to both nucleus and cytoplasm (30). Hence to be present at different locations and to bind to a large number of transcripts it has to be translated more often and should be present in a higher number of copies. Consistent with this, we find that its transcript exhibits high ribosome occupancy. Indeed, Hogan et al. (23) demonstrated that RNA targets of highly connected RBPs were enriched for multiple processes and subcellular localizations. These results clearly unveil the strong relationship between the concentration of a RBP and the number of distinct RNA targets bound by them, indicating that RBPs responsible for controlling a wide range of targets must occur in a higher number of copies at the protein level. It is important to note that although RBPs as a group of genes are significantly higher expressed at the transcript and protein levels compared to non-RBP population, relative abundance of the RBPs is correlated to the hierarchy of a RBP, defined as the number of distinct RNA targets. It is also noteworthy to mention that the RBPs analyzed for connectivity in this section did not comprise core ribosomal proteins, strengthening the generality of these observations.

RBPs Bound to Many RNA Targets Are Less Frequently Degraded and Tightly Controlled at Protein Level. Although RBPs with a higher number of distinct targets are expressed at a higher level compared to those that control fewer targets, it is not evident whether their protein turnover rates would hold a similar trend. Therefore, to understand whether there is any dependence between the stability of a RBP and the number of transcripts it controls, we used a similar

and quantified them using microarray analysis. mRNA copy number could be obtained for 5,643 genes whereas ribosome occupancy could be mapped for 5,700 genes, allowing us to study the extent of transcript abundance and translation rates of the genes and transcripts. Stability of a protein, which is an estimate of the duration it occurs within the cell is measured as the half-life of the protein. In yeast, protein half-lives have been estimated by Belle and coworkers for $\approx 3,750$ proteins by inhibiting translation (41). In this study, we used these data by excluding proteins whose half-lives have been obtained by extrapolation. Protein abundance, which reveals the absolute number of protein molecules per cell, was obtained from Ghaemmaghami et al. (42). We could obtain abundance values for 3,868 proteins in the entire genome. Biological noise, which is typically defined as the variation in the expression of a protein between different cells in a homogenous population of cells, was obtained from Newman et al. (25). We could obtain noise data for 2,213 genes for cells grown on rich media. The authors in this study used two distinct measures for calculating protein noise, coefficient of variation (CV), which is the ratio of the standard deviation in the expression of a protein and its mean expression and distance from median (DM), which was calculated as the difference between the CV value of a protein and a running median of all CV values. In this study, we have used DM as a measure of protein noise as it was indicated to be a more robust measure compared to CV to understand protein-to-protein variations in noise levels (25). Since DM is the distance between the CV and median value of all CVs, negative values correspond to relatively less noise whereas positive values reflect higher levels of noise in the protein expression.

Comparison of the Regulatory Properties of RBPs with Other Protein Coding Genes. To study whether RBPs show differences in dynamic properties when compared to other protein coding genes, we defined a non-RBP set of proteins. This set essentially comprised proteins in the whole genome after excluding the list of 561 RBPs defined above. To assess whether RBPs exhibit a different trend compared to non-RBPs for each of the properties studied, we used the Wilcoxon rank-sum test or the Mann–Whitney U test available in the *R* statistical package to calculate the significance. The Wilcoxon test enables the comparison of two samples to assess whether they come from the same distribution or not. Since this

test is nonparametric and does not assume any inherent distribution of the samples it is ideal to compare different samples. Box plots were used to represent the distribution of values for each property. Independently, analysis of the mean values of a property for RBP and non-RBP sets of proteins was also carried out and *P*-values were estimated using the Welch *t* test, which gave similar results (SI Text S5). Because the RBP set comprised a number of ribosome-associated proteins we also excluded them from this list and repeated the analysis to test the robustness of the tendencies observed, in the absence of ribosomal proteins (SI Text S2).

Analysis of the Relationship Between the Number of Targets of a RBP and Their Dynamic Properties. To understand the link between the number of targets of a RBP and their dynamic properties, RBPs were first grouped on the basis of their number of distinct RNA targets to which they were bound. This grouping was done in such a way that each bin of RBPs contained roughly an equal number of RBPs. This resulted in five different bins corresponding to varying degrees of RBPs, with some RBPs controlling as many as 2,000 mRNAs in the RBP-RNA network. To nullify the effect of outliers in each bin, median values were calculated for different dynamic properties and correlation was estimated between median values and connectivity of RBPs. *P*-values were calculated using the coefficient of correlation and the number of data points, on the basis of a linear fit.

Supporting Information. For additional details relating to *SI Text S1–S6*, see *Dataset S1*, *Figs. S1–S7*, and *Tables S1–S3*. An analysis of enrichment of cell-cycle regulated genes, performed to address the concerns of a reviewer, is detailed in *SI Text S7*.

ACKNOWLEDGMENTS. We thank members of the Theoretical and Computational Biology group at MRC Laboratory of Molecular Biology (LMB) for their feedback and helpful discussions in the early stages of this work. We also thank S. De, B. Lang, Y. Kondo, and Y. Pilpel for providing helpful comments. This work was supported by MRC LMB (to N.M., S.C.J., and M.M.B.), by National Institute of Pharmaceutical Education and Research, Commonwealth split-site program (to N.M., N.R., and M.M.B.) and Cambridge Research Commonwealth Trust (to S.C.J.).

Supplementary URL: <http://www.mrc-lmb.cam.ac.uk/genomes/sarath/RBP-dynamics>

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